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Yersinia pestis YopD 150–287 fragment is partially unfolded in the native state ☆

Ronald Raab [a], Wieslaw Swietnicki [b,*]

[a] *ISAT, James Madison University, Harrisburg, VA, USA*
[b] *USAMRIID, Division of Bacteriology, Fort Detrick, MD 21702, USA*

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Abstract

Yersinia pestis, a human and animal pathogen, uses the type III secretion system (T3SS) for delivering virulence factors and effectors into the host cells. The system is conserved in animal pathogens and is hypothesized to deliver the virulence factors directly from bacterial to mammalian cells through a pore composed of YopB and YopD translocation proteins. The YopB and YopD translocator proteins must be delivered first to form a functional pore in the mammalian cell. The criteria by which *Yersinia* selects the two proteins for initial delivery are not known and we hypothesized that the extensive binding by the chaperone and partial unfolding of the unbound region may be the criteria for selection. The YopB and YopD translocator proteins, unlike other effectors, have a common chaperone SycD, which binds through multiple regions. Due to the small size of the pore, we hypothesized that many of the transported virulence factors, translocators YopB and YopD included, are delivered in a partially unfolded state stabilized by binding to specific chaperones. The YopD protein binds the chaperone through amino acid (a.a.) 53–149 and a.a. 278–292 regions but biophysical characterization of YopD has not been possible due to the lack of an expression system for soluble, large fragments of the protein. In our present work, we demonstrated that the YopD 150–287 peptide fragment, almost the full soluble C-terminal part, including the non-interacting peptide fragment YopD 150–277, was partially unfolded in its native state by a combination of biophysical methods: circular dichroism, quasi-elastic light scattering, chemical unfolding and 8-anilino-1-naphthalene sulfonate (ANS) binding. The secondary structure of the peptide converted easily between α-helical and random coil states at neutral pH, and the α-helical state was almost fully recovered by lowering the temperature to 263 K. The current results suggest that YopD 150–287 peptide may have the postulated transport-competent state in its native form.

Keywords: *Yersinia pestis*; YopD; SycD; Translocator; Molten globule

Yersinia pestis is a human and animal pathogen with the type III secretion system for delivering virulence factors from the bacterial cytosol into the mammalian cell (review in [1]). The transport is directional and requires cell contact and pore formation [2,3] before injection of the virulence factors. Two translocators, YopD and YopB, are postulated to form a pore in the mammalian cell membrane [4] through which all other effectors are delivered. The criteria by which *Y. pestis* selects the YopB and YopD translocators to be delivered first are not known. The LcrV protein is localized on the tip of needle [5] and most likely is not in the pore, similar to the data for *Pseudomonas aeruginosa* ortholog PcrV [6], as it lacks a defined transmembrane domain. It is not excluded, however, that it may be associated with a pore structure. The delivery of effectors is most likely through this polymerized needle, composed mainly of YscF in *Yersinia* [7]. The energy to transport the factors may come from ATP hydrolysis, similar to the enteropathogenic *Escherichia coli* type III secretion system [8]. The pore diameter, as measured by the solute diffusion method,

☆ Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the US Army.

* Corresponding author. Fax: +1 301 619 2348.
E-mail addresses: raabrw@cisat.jmu.edu (R. Raab), wes.swietnicki@amedd.army.mil (W. Swietnicki).

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14. ABSTRACT

Yersinia pestis is a human and animal pathogen uses the type III secretion system (TTSS) for delivering virulence factors and effectors into the host cells. The system is conserved in animal pathogens and is hypothesized to deliver the virulence factors directly from bacterial to mammalian cells through a pore composed of YopB and YopD translocation proteins. The YopB and YopD effector proteins must be delivered first to form a functional pore in the mammalian cell. The criteria by which Yersinia selects the two proteins for initial delivery are not known and we hypothesized that the extensive binding by the chaperone and partial unfolding of the unbound region may be the criteria for selection. The YopB and YopD proteins, unlike other effectors, have a common chaperone SycD, which binds through multiple regions. Due to the small size of the pore, we hypothesized that many of the transported virulence factors, YopB and YopD included, are delivered in a partially unfolded state stabilized by binding to specific chaperones. The YopD protein binds the chaperone through amino acid (a.a.) 53-149 and a.a. 278-292 regions but biophysical characterization of YopD has not been possible due to the lack of an expression system for soluble, large fragments of the protein. In our present work, we demonstrated that the YopD 150-287 peptide fragment, almost the full soluble C-terminal part, including the non-interacting peptide fragment YopD150-277, was partially unfolded in its native state by a combination of biophysical methods: circular dichroism, quasi-elastic light scattering, chemical unfolding and 8-anilino-1-naphtalene sulfonate (ANS) binding. The secondary structure of the peptide converted easily between alpha-helical and random coil states at neutral pH, and the alpha-helical state was almost fully recovered by lowering the temperature to 262°K. The current results suggest that YopD 150-287 peptide may have the postulated transport-competent state in its native form.

15. SUBJECT TERMS
Yersina pestis, plague, type III secretion system, YopD, virulence factors

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has been estimated at 2–3 nm [9]. A recent model of polymerized MxiH needle protein from *Shigella flexneri* confirmed these estimates [10]. Because many of the effectors, e.g., YpkA, YopH from *Y. pestis*, are over 450 amino acids (a.a.)[1] long, the diameter of the pore is not large enough to transport the fully folded protein. As an alternative explanation, it has been postulated that the effector proteins are partially unfolded, with the help of a specific chaperone, before transport. The hypothesis is supported by crystal structures of the Sip–SptP (1–158) complex, which shows a partially unfolded effector [11]. Similar observations have been confirmed in other complexes of effector fragments and chaperones, suggesting that the effector fragments prefer extended conformations when binding to chaperones [12–14]. In agreement with the previous data, it has been shown recently that the binding of the SycN–YscB complex to the secretion substrate YopN is through an extended conformation with some secondary but no tertiary structure [15].

The *Y. pestis* YopD (306 a.a.) and YopB (401 a.a.) translocators are putative transmembrane proteins with two potential transmembrane regions: a.a. 166–184 and 228–246 in YopB, and a.a. 75–93 and 128–149 in YopD. The proteins are quickly degraded in bacteria when not bound to its common chaperone SycD [16]. The SycD binding region was localized to the N-terminal a.a. 53–149 and C-terminal a.a. 278–292 fragments of YopD by a yeast two-hybrid system [17]. Binding of SycD to YopB protein was found in many discreet regions: a.a. 56–99, 172–210 and 329–395, almost a half of the protein length [16]. The binding arrangements for both translocators are unique among other effectors, which have single chaperone with a much-defined binding region. The full-length and N-terminal binding regions of YopD are insoluble and *in vitro* biophysical characterization has not been achieved. To the contrary, the C-terminal YopD 278–292 peptide is readily soluble and forms an amphipathic α-helix in solution [18]. The binding site of YopD was postulated to localize to a large surface on the model of SycD, the opposite side of the YopB interactions [19], in agreement with data for a common chaperone of YopD and YopB [16].

To help us understand the preferential selection of YopB and YopD over other effectors in the delivery process, we hypothesized that requirements may be related to the biophysical properties of the proteins. A likely option would be an extensive coverage by SycD chaperone, as already reported, and an increased degree of flexibility of the unbound fragments in the native state, as reported in the current work, to bypass the energy requirement for partial unfolding. In the case of YopD, the only fragments not interacting with the chaperone are the N-terminal a.a. 1–52 and C-terminal a.a. 150–277 and a.a. 293–306 regions [17]. The N-terminal region is a long peptide and much unstructured (W. Swietnicki, unpublished data) and the second C-terminal region is too short to have a stable tertiary structure. On the contrary, the first C-terminal region is predicted to be highly α-helical and therefore was chosen for the current study. The YopD 150–287 fragment, almost the full C-terminal 150–306 region, was expressed in two different systems: as a refolded species and as a native species. The secondary structure of both proteins was identical. The refolded protein was studied by various biophysical methods and was partially unfolded. Also, the secondary structure of the protein easily converted between α-helical and random coil states at neutral pH. The current work demonstrates that a large soluble fragment YopD 150–287 has a highly flexible conformation in its native state. Together with other data, the finding may offer an alternative explanation for selection of YopD over other, non-translocator, virulence factors in the delivery by *Y. pestis* T3SS. Also, the finding adds YopD to a growing list of intrinsically disordered proteins in their native states [20–22].

Materials and methods

Gene cloning, protein expression and purification

The YopD 150–287 fragment was cloned into two bacterial expression systems: the N-terminal intein fusion expression vector pTYB12 (New England Biolabs) and an N-terminal 6xHis fusion vector RSF-2 Ek/LIC (Novagen).

For the intein expression system, the DNA region corresponding to YopD a.a. 150–287 was cloned from genomic DNA by PCR, restricted with NdeI/XhoI, and ligated into a predigested pTBY12 (New England Biolabs) vector. The clones were screened by restriction digest with HindIII and positive colonies were submitted for DNA sequencing. The final construct coded for a maltose-binding protein domain followed by an intein and the YopD 150–287 fragment. The construct was transformed into *E. coli* BL21 (DE3) strain for protein expression. The final construct was designed to code for YopD 150–287 only after cleavage by intein. To purify protein, a cell paste from 50 ml of 37 °C culture induced with 0.5 mM IPTG for 3–6 h at room temperature was resuspended in column buffer provided with the IMPACT system (New England Biolabs), disrupted by sonication, spun at 16,000*g* to remove cellular debris, and purified as suggested by the IMPACT system manual. The purified protein was dialyzed against 10 mM Tris, pH 7.0, 150 mM NaCl and concentrated to 0.4 mg/ml. The preparation was distributed to aliquots and stored at −80 °C.

The RSF-2 construct was amplified from a genomic DNA according to manufacturer's instructions, sequenced in full and transformed into the Rosetta (DE3) *E. coli*

[1] *Abbreviations used:* a.a., amino acid; ANS, 8-anilino-1-naphthalene sulfonic acid; CD, circular dichroism; GuHCl, guanidine hydrochloride; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PBS, phosphate buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, Tris(hydroxymethyl)aminomethane; *Y. pestis*, *Yersinia pestis*.

strain (Novagen) for protein expression. The final construct coded for the YopD 150–287 fragment preceded by an enterokinase cleavage site and a 6xHis affinity tag. For the purpose of cloning and protein expression, a Met residue was added to the start of the reading frame.

The protein was expressed overnight in a Terrific Broth medium supplemented with kanamycin (50 μg/ml) at 30 °C after a 40-fold dilution of a 25 ml starter culture grown at 37 °C. Cells were harvested by centrifugation, resuspended in 1/10 volume of refolding buffer (6 M GuHCl, 100 mM potassium phosphate, pH 8.0) supplemented with protease inhibitors and disrupted by sonication. The crude lysate was centrifuged for 2 h at 30,000*g* at 4 °C and the supernatant was loaded onto the pre-equilibrated Ni-agarose column. The refolding was accomplished as described previously [23] and the protein was polished by a combination of ion exchange on a HiPrep 16/10 Q Sepharose (GE Healthcare) column, gel filtration on a HiLoad 16/60 Superdex 200 (GE Healthcare) column, and a hydrophobic interaction chromatography on a HiLoad 16/10 Phenyl Sepharose HP (GE Healthcare) column. The final preparation was dialyzed against 10 mM Tris–HCl, pH 8.0, 0.3 M NaCl and concentrated to 13.0 mg/ml as determined spectrophotometrically. Protein was distributed to vials and stored at −80 °C. A fresh aliquot was used for experiments.

CD measurements

For the far-UV measurements, the protein was diluted to 0.2–0.4 mg/ml in 10 mM Tris, pH 8.0, 150 mM NaCl and 10 spectra in the 200–250 nm range were collected at room temperature using the 50 nm/min scanning speed, 1 s time constant and 0.2 nm resolution. The spectra were converted to molar ellipticity per residue and plotted with the KaleidaGraph (Synergy Software) program.

The temperature scan of far-UV spectra of protein was performed as described above with two modifications: 30% glycerol (v/v) was added to prevent freezing the solution below 0 °C and the buffer was changed to 1× PBS to prevent pH variation with temperature observed for Tris-based buffers.

The near-UV measurements were performed in a 1 cm path length cuvette at 0.893 mg/ml concentration in a 1× PBS buffer. Typically, 10 spectra were collected at room temperature in the 250–300 nm range at 50 nm/min scanning speed, 1 s time constant, and 1 nm resolution, averaged, converted to molar ellipticity, and plotted with the KaleidaGraph (Synergy Software) program.

The chemical unfolding experiments were performed on a Jasco 810 (JASCO) spectropolarimeter at room temperature with an automatic titrator attachment (JASCO) as described [23] with the following modification: four instead of two spectra were collected and averaged. The data were converted to molar ellipticity per residue and plotted versus denaturant concentration with KaleidaGraph (Synergy Software) software.

Prediction of secondary structure and transmembrane regions

Secondary structure predictions were based on sequence analysis using the consensus secondary structure value obtained from a Network Protein Sequence Analysis bioinformatics server (http://npsa-pbil.ibcp.fr). A default parameter set included DPM [24], DSC [25], GORIV [26], HNN [27], PHD [28], PREDATOR [29], SIMPA96 [30] and SOPM [31] prediction methods. Calculations did not include the leader sequence from the vector. Transmembrane regions were predicted by the TMPRED program at the EMBnet bioinformatics server (http://www.ch.embnet.org). The program uses statistical analysis of a database of annotated transmembrane proteins [32].

8-Anilino-1-naphthalene sulfonic acid (ANS) binding

The experiments were performed at room temperature on FP-6500 (JASCO) spectrofluorometer. A 10 mM solution of ANS was added to a 300 μL sample of protein at 0.4 mg/ml in 1× PBS excited at 395 nm. After each addition, four emission spectra in the 420–600 nm range were collected and corrected for the buffer. The instrument parameters were: 3 nm emission and excitation band widths, scanning speed 200 nm/min, 0.5 s time constant, and 1 nm resolution. The spectra were corrected for dilution of ANS and plotted with the KaleidaGraph (Synergy Software) software.

Quasi-elastic light scattering

The experiments were performed on a Dyna Pro (Wyatt Technologies) instrument by measuring the right angle dynamic light scattering at room temperature. The protein sample, about 80 μL, was prepared in 10 mM Tris, pH 8.0, 150 mM NaCl, at 6 mg/ml, spun at 16,000*g* for 5 min and filtered through a 100 nm filter. The final solution was introduced in the cell and spectra were collected until the signal was stabilized, approximately 2–3 min. The data were analyzed, assuming a globular shape of the species, for size distribution in the regularization mode according to instruments software.

Results

In our study, we initially observed that the YopD 150–287 peptide had a molten globule-like conformation in its native state based on the following criteria: absence of a defined transition region in the chemical unfolding monitored by CD, absence of a fingerprint signature in the near-UV CD spectrum, increased hydrodynamic radius as estimated by quasi-elastic light scattering, and a large increase in ANS fluorescence upon binding to the protein. The secondary structure of the protein could easily convert between α-helical and random coil states at neutral pH by changing temperature.

To study the biophysical properties of the YopD 150–287 fragment, the protein was made in bacterial systems using native and refolding conditions and characterized in more detail in the current work. Other peptides from YopD were either insoluble or too short to have a tertiary structure (W. Swietnicki, unpublished data).

The overall purification strategy and yields are presented in Table 1. The refolded version expressed at much higher yields than the one purified under native conditions and was suitable for a thorough cleanup by a combination of affinity, ion exchange, gel filtration and hydrophobic interaction chromatography. The native protein was expressed at much lower yields and, therefore, was used mainly for a reference in circular dichroism experiments. The purity of the recombinant proteins was more than 90% as judged by SDS–PAGE (Fig. 1) which was suitable for the more detailed biophysical characterization. The far-UV CD spectrum of recombinant YopD 150–287 peptide is a combination of α-helix and random coil as predicted (Fig. 2). The spectra of refolded and native proteins are perfectly superimposable in the 202–250 nm regions, which confirm that the secondary structures of the refolded and native proteins were identical. Because the secondary structure of the refolded YopD 150–287 peptide was identical to the native YopD 150–287 peptide, all other experiments were performed with the refolded peptide.

The estimate of α-helical content is 0.26–0.29, about a third of the predicted value (Table 2). The discrepancy is much larger than typically attributed to prediction errors and we hypothesized that there might be an alternative explanation. To investigate the issue, we measured the far-UV spectra at lower temperatures, hoping that the secondary structure was more stable. As hypothesized, at lower temperatures the secondary structure became enriched in α-helix at the expense of random coil (Fig. 3A). The conversion was gradual and at 263 K the measured α-helical content reached almost 90% of the predicted value (Fig. 3B). The data are consistent with a molten globule-like state as opposed to a sharp transition observed in a two-state conformational equilibrium [33].

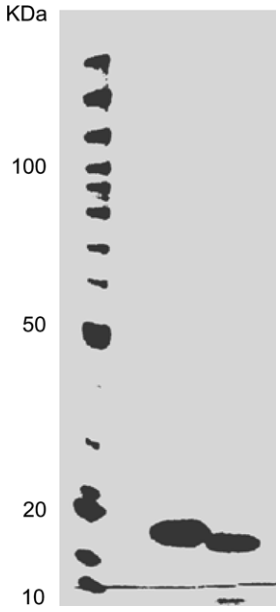

Fig. 1. Purity of the purified YopD 150–287 peptides by SDS–PAGE. Lanes: 1, M.W. markers; 2, refolded YopD 150–287; 3, native YopD 150–287. Approximately 2 μg of refolded and 1.4 μg of native peptides were loaded per lane. Electrophoresis was run on 4–12% Bis–Tris Novex gel (Invitrogen) in 1× MOPS buffer and visualized using SafeBlue (Invitrogen) solution according to manufacturer's instructions.

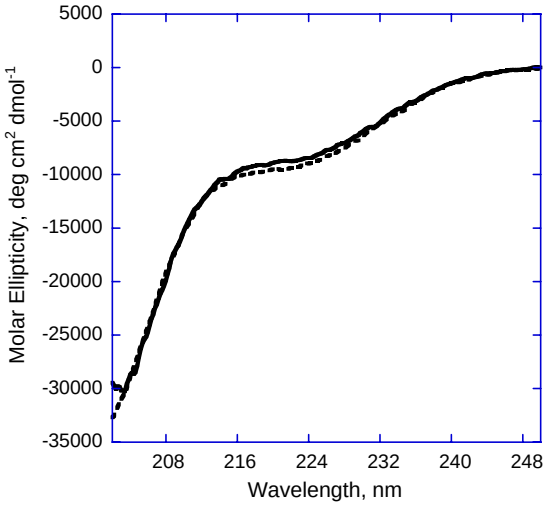

Fig. 2. Far-UV circular dichroism spectra of YopD 150–287 peptide. The spectra were taken in 10 mM Tris, pH 8.0 for the refolded (solid line) and native (dashed line) peptides.

Table 1
Summary of purification of YopD 150–287 polypeptide

Step	Total protein (mg)	Total yield (%)	Purity (%)
Refolded	>100[a]	100	<10
E. coli	66[b]	66	>50
Ni-agarose	31[b]	31	>90
Ion exchange	31[b]	31	>90
Gel filtration	13[b]	13	>99
Hydrophobic interactions	13[b]	13	>99
Concentrate	13[b]		
Native			
E. coli	15	10	<10
Chitin resin	13[b]	87	>80

[a] The number corresponds to the total weight of the harvested cells from 1 L of culture.
[b] Total protein content was determined spectrophotometrically based on calculated amino acid composition of the purified YopD 150–287 protein.

The near-UV CD spectrum is composed of contributions from aromatic residues (Trp, Phe, Tyr) and indicates tertiary structure in the protein. The YopD 150–287 peptide near-UV CD spectrum (Fig. 4) had minimal signal in the region, which was mostly absent in 4 M GuHCl, concentration sufficient to unfold most of the α-helix (discussed later). The small absolute value may be related to partial peptide aggregation due to exposed hydrophobic

Table 2
Estimates of α-helical content in YopD 150–287 polypeptides

Peptide	Fraction of α-helical content	
	Predicted[a]	Experimental[b]
Native	0.80	0.29
Refolded	0.81	0.26

[a] Predictions were made as described in Materials and methods.
[b] Molecular ellipticity per residue value at 222 nm was calculated according to the formula: % α-helix = $\Theta_{222}/3298 * (-10)$ [39].

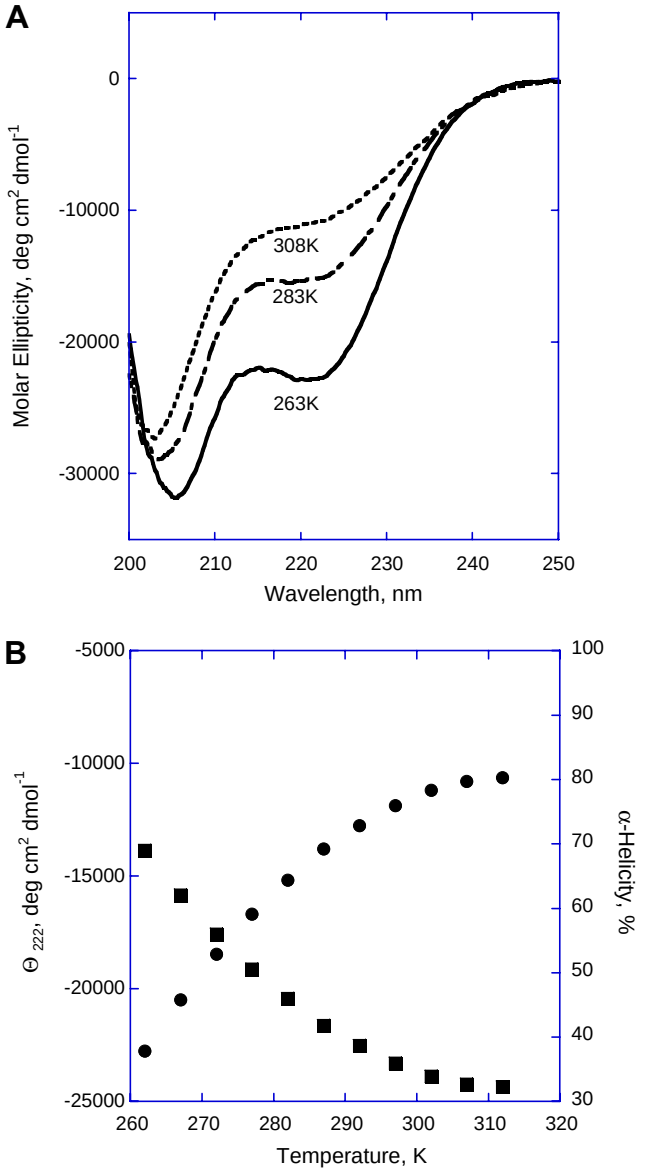

Fig. 3. Conformational transitions between α-helix and random coil in YopD 150–287 peptide as observed by far-UV circular dichroism. (A) Spectra of YopD at 263, 283 and 308 K. (B) Temperature dependence of a measured α-helical signal at 222 nm (left axis, filled circles) and corresponding calculated α-helical content (right axis, filled squares). The measurements were performed in 1× PBS and 30% (v/v) glycerol.

surfaces (discussed later). The peptide then has practically no tertiary structure as observed for a molten globule state [34].

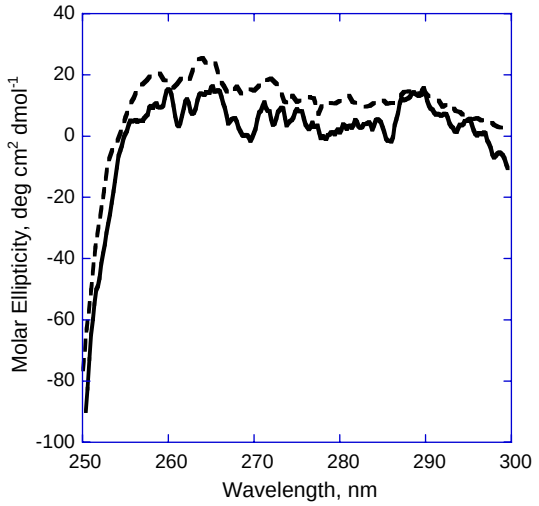

Fig. 4. Absence of tertiary structure in YopD 150–287 as measured by near-UV circular dichroism. The spectrum of refolded peptide was determined in (dashed line) 10 mM Tris, pH 8.0, 150 mM NaCl or (solid line) 4 M GuHCl, 10 mM Tris, pH 8.0, 150 mM NaCl.

The molten globule structure is associated with increased hydrophobic radius as compared to a compact, globular protein. The measurement of a diffusion coefficient, indicating hydrodynamic radius of the protein, by quasi-elastic light scattering, determined the hydrodynamic radius at 3.15–3.23 nm (Table 3). The value corresponded to a molecular mass of 49–52 kDa for a globular protein and was about three times the mass value for the YopD 150–287 peptide. However, the hydrodynamic radius associated with molten globule state is about 10% higher than for a compact state and the YopD 150–287 peptide is most likely a trimeric species or is in an extended conformation. The last possibility is favored by chemical unfolding and ANS binding experiments (discussed later).

The chemical unfolding of the protein is another parameter for molten globule-like states. The YopD 150–287 peptide had no sharply defined transition region when unfolded by guanidine hydrochloride at neutral pH at room temperature (Fig. 5). The tertiary structure of the peptide was then absent as observed for molten globule-like states.

The molten globule-like states had an increased percentage of hydrophobic surfaces exposed to the solvent. Binding of fluorescent dyes, ANS or bis-ANS, causes a large increase in fluorescence emission of the dye due to binding

Table 3
Molecular radius determination of YopD 150–287 peptide as determined by quasi-elastic light scattering

Diffusion coefficient × 10^{-9} (cm^2/s)	MW (kDa)	Radius (nm)
751–774	49–52	3.15–3.23

Data were collected at room temperature and analyzed as described in Materials and methods on DynaPro Protein (Wyatt Technologies) instrument. The lower and higher values in the range correspond to different regularization protocols during data analysis.

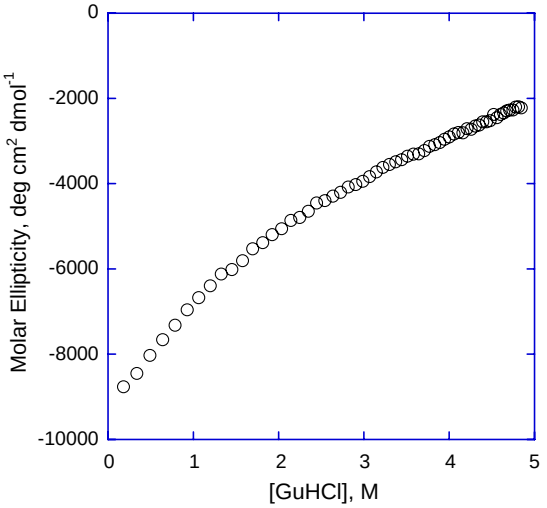

Fig. 5. Stability of YopD 150–287 peptide against chemical method as monitored by circular dichroism. (A) The chemical unfolding in 1× PBS by GuHCl as monitored by Θ_{222}. Peptide concentration was 0.02 mg/ml.

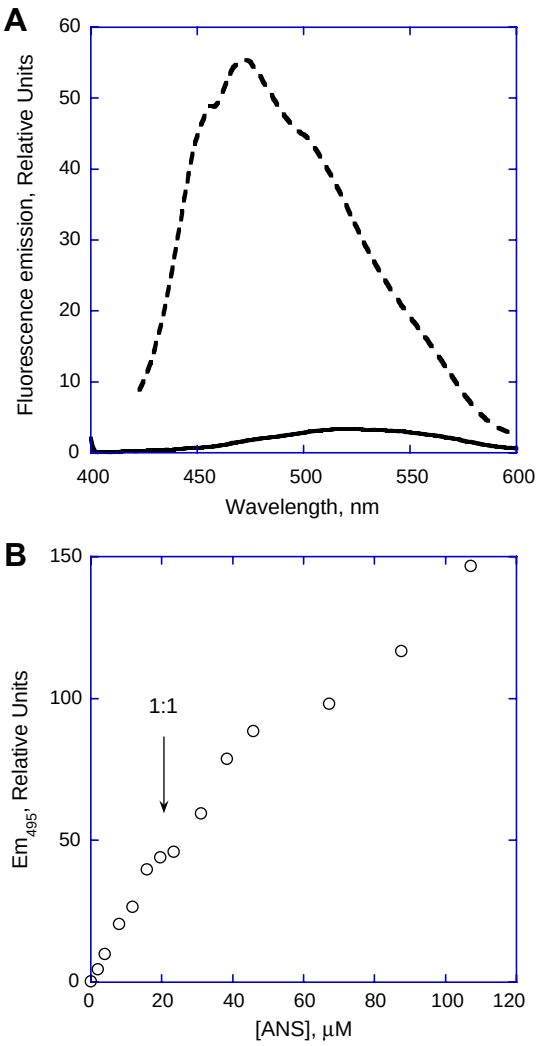

Fig. 6. Exposure of hydrophobic regions in YopD 150–287 under neutral conditions as monitored by ANS binding. (A) Fluorescence emission spectra of ANS in the absence (solid line) and presence (broken line) of peptide. Peptide and ANS concentrations were approximately 20 μM in 1× PBS. (B) Binding of ANS to YopD 150–287 as monitored by ANS fluorescence emission at 495 nm. Peptide concentration was 23 μM in 1× PBS. Arrow corresponds to approximately 1:1 ANS–peptide ratio. The data were corrected for the scatter of the buffer.

to those surfaces [35,36]. In YopD 150–287, the increase was at least 20-fold (Fig. 6A), consistent with exposed hydrophobic areas. Titration of the peptide with ANS identified multiple discrete binding domains (Fig. 6B), which indicated multiple hydrophobic exposed surfaces. The binding was not saturable and, therefore, the affinity and stoichiometry of the process was not studied. Overall, the behavior is consistent with a partially unfolded peptide.

The YopD 150–287 peptide contains the first 10 residues of the region proposed to bind the SycD chaperone [18]. It was of interest to investigate if the truncated region was sufficient to bind the SycD chaperone in a stable complex. Unfortunately, co-expression of both proteins from the same vector in many systems did not produce stable complexes (W. Swietnicki, data not shown). The shorter region was then not sufficient to form a stable complex with the SycD chaperone.

Discussion

We demonstrated that almost the full C-terminal part of YopD effector, YopD 150–287 fragment, was partially unfolded by a combination of near- and far-UV CD spectroscopy, chemical stability, quasi-electric light scattering, and ANS binding. The secondary structure of the peptide converted easily between α-helical and random coil states at neutral pH.

The detailed study of SycD–YopD interactions *in vitro* has not been possible due to insolubility of the YopD protein. Therefore, the requirements for potential unfolding of the rest of the protein, as postulated for the transport through the pore, are not known. In this study, we found that the large YopD 150–287 peptide, almost the full C-terminal region, was soluble and partially unfolded in its native state, as required for the transport. To study this peptide in detail, we purified the peptide by on-column refolding to a high yield (100 mg/L of culture expression level) and, for comparison, under native conditions in the intein system to a low yield (13–15 mg/L of culture expression level) as shown in Table 1. The purity of both proteins was over 90% as judged by SDS–PAGE (Fig. 1), sufficient for a more detailed characterization. Because both peptides had identical secondary structure (Fig. 2), the contribution of potential folding artifacts to the secondary structure of the refolded peptide was negligible. Therefore, detailed studies were performed with the high-yield refolded peptide. The peptide secondary structure, a combination of α-helix and random coil, is about a third of the predicted value (Table 2). The discrepancy is due to the highly flexible secondary structure of the peptide (Fig. 3B) as 90% of the predicted α-helical content could be recovered by low-

ering the temperature to 262 K (Fig. 3B). The fingerprint signature in the near-UV region, characteristic of a tertiary structure, is absent (Fig. 4), as observed in molten globule states. Consequently, the peptide lacks a sharp transition region in the chemical unfolding (Fig. 5) and has an increased hydrodynamic radius (Table 3). The peptide flexibility, as determined by chemical unfolding, is most likely caused by partial unfolding, which exposed hydrophobic surfaces (Fig. 6A).

The YopD 150–287 peptide contains part of the SycD chaperone site and lacks a defined tertiary structure, as observed in many chaperone–effector fragment complexes. The fragment, however, was not sufficient to form a stable complex when co-expressed with SycD chaperone (W. Swietnicki, data not shown), despite the rest of the peptide being very flexible and hydrophobic. The chaperone binding, then, required at least the full C-terminal binding site *in vitro*, inclusion of which made the YopD protein insoluble (W. Swietnicki, unpublished data). The full N-terminal chaperone binding site on YopD detected in an *in vivo* screen, including the residues in the transmembrane region [17], could not be studied *in vitro* as those fragments were insoluble (W. Swietnicki and R. Raab, unpublished data).

In *P. aeruginosa*, the functionally equivalent YopB and YopD orthologs, PopB and PopD, form ring-like structures *in vitro* in artificial membranes, and are both required for membrane permeabilization [37]. The membrane insertion of both proteins increased with decreasing pH, in agreement with SycD ortholog PcrH release [38]. In *Y. pestis*, low pH caused irreversible unfolding of the YopD 1–75 and 150–306 fragments (W. Swietnicki, unpublished data). It is possible partial destabilization of the YopD translocator by lowering pH may have a physiological role.

The partial unfolding of the YopD 150–287 fragment may be of importance when considering the transport of YopD protein through the translocation pore. The YopD 56–149 and 278–292 regions formed a complex with SycD chaperone in the bacterial cytoplasm and were already in a translocation competent state. Of the remaining regions, the YopD 150–287 fragment was the largest, corresponding to almost the full C-terminal domain, and was in a flexible conformation as observed for effector fragments–chaperone complexes. The full-length YopD protein may then require less energy to transport due to the partial unfolding of the 150–287 region and therefore may reach the mammalian cell faster than other effectors. Also, the partial unfolding of the region may be advantageous when the protein is, presumably, inserted in mammalian cell membranes, similar to the mechanism proposed for the *P. aeruginosa* PopB and PopD translocators [38]. Analogous experiments for the *Y. pestis* YopB protein would be of interest to confirm the hypothesis.

In summary, we expressed almost the full C-terminal part of YopD protein, YopD 150–287, and studied its properties in solution. The peptide was very flexible under neutral pH conditions but had a defined secondary structure. The flexible conformation of the peptide in the absence of SycD chaperone may be of an advantage when considering the secretion order by *Y. pestis* type III secretion system and/or mammalian cell membrane insertion.

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References

[1] G.R. Cornelis, The Yersinia Yop virulon a bacterial system to subvert cells of the primary host defense, Folia Microbiol. (Praha) 43 (1998) 253–261.
[2] D. Buttner, U. Bonas, Port of entry—the type III secretion translocon, Trends Microbiol. 10 (2002) 186–192.
[3] B.K. Coombes, B.B. Finlay, Insertion of the bacterial type III translocon: not your average needle stick, Trends Microbiol. 13 (2005) 92–95.
[4] M.S. Francis, H. Wolf-Watz, YopD of Yersinia pseudotuberculosis is translocated into the cytosol of HeLa epithelial cells: evidence of a structural domain necessary for translocation, Mol. Microbiol. 29 (1998) 799–813.
[5] C.A. Mueller, P. Broz, S.A. Muller, P. Ringler, F. Erne-Brand, I. Sorg, M. Kuhn, A. Engel, G.R. Cornelis, The V-antigen of Yersinia forms a distinct structure at the tip of injectisome needles, Science 310 (2005) 674–676.
[6] J. Goure, A. Pastor, E. Faudry, J. Chabert, A. Dessen, I. Attree, The V antigen of *Pseudomonas aeruginosa* is required for assembly of the functional PopB/PopD translocation pore in host cell membranes, Infect. Immun. 72 (2004) 4741–4750.
[7] E. Hoiczyk, G. Blobel, Polymerization of a single protein of the pathogen Yersinia enterocolitica into needles punctures eukaryotic cells, Proc. Natl. Acad. Sci. USA 98 (2001) 4669–4674.
[8] R. Zarivach, M. Vuckovic, W. Deng, B.B. Finlay, N.C. Strynadka, Structural analysis of a prototypical ATPase from the type III secretion system, Nat. Struct. Mol. Biol. 14 (2007) 131–137.
[9] A. Holmstrom, J. Olsson, P. Cherepanov, E. Maier, R. Nordfelth, J. Pettersson, R. Benz, H. Wolf-Watz, A. Forsberg, LcrV is a channel size-determining component of the Yop effector translocon of Yersinia, Mol. Microbiol. 39 (2001) 620–632.
[10] J.E. Deane, P. Roversi, F.S. Cordes, S. Johnson, R. Kenjale, S. Daniell, F. Booy, W.D. Picking, W.L. Picking, A.J. Blocker, S.M. Lea, Molecular model of a type III secretion system needle: Implications for host-cell sensing, Proc. Natl. Acad. Sci. USA 103 (2006) 12529–12533.
[11] C.E. Stebbins, J.E. Galan, Maintenance of an unfolded polypeptide by a cognate chaperone in bacterial type III secretion, Nature 414 (2001) 77–81.
[12] C.B. Trame, D.B. McKay, Structure of the Yersinia enterocolitica molecular-chaperone protein SycE, Acta Crystallogr. D Biol. Crystallogr. 59 (2003) 389–392.
[13] S.C. Birtalan, R.M. Phillips, P. Ghosh, Three-dimensional secretion signals in chaperone–effector complexes of bacterial pathogens, Mol. Cell 9 (2002) 971–980.
[14] M. Lilic, M. Vujanac, C.E. Stebbins, A common structural motif in the binding of virulence factors to bacterial secretion chaperones, Mol. Cell 21 (2006) 653–664.
[15] F.D. Schubot, M.W. Jackson, K.J. Penrose, S. Cherry, J.E. Tropea, J.V. Plano, D.S. Waugh, Three-dimensional structure of a macromolecular assembly that regulates type III secretion in *Yersinia pestis*, J. Mol. Biol. 346 (2005) 1147–1161.
[16] C. Neyt, G.R. Cornelis, Role of SycD, the chaperone of the Yersinia Yop translocators YopB and YopD, Mol. Microbiol. 31 (1999) 143–156.

[17] M.S. Francis, M. Aili, M.L. Wiklund, H. Wolf-Watz, A study of the YopD–lcrH interaction from Yersinia pseudotuberculosis reveals a role for hydrophobic residues within the amphipathic domain of YopD, Mol. Microbiol. 38 (2000) 85–102.
[18] T. Tengel, I. Sethson, M.S. Francis, Conformational analysis by CD and NMR spectroscopy of a peptide encompassing the amphipathic domain of YopD from Yersinia, Eur. J. Biochem. 269 (2002) 3659–3668.
[19] P.J. Edqvist, J.E. Broms, H.J. Betts, A. Forsberg, M.J. Pallen, M.S. Francis, Tetratricopeptide repeats in the type III secretion chaperone, LcrH: their role in substrate binding and secretion, Mol. Microbiol. 59 (2006) 31–44.
[20] D.P. Denning, S.S. Patel, V. Uversky, A.L. Fink, M. Rexach, Disorder in the nuclear pore complex: the FG repeat regions of nucleoporins are natively unfolded, Proc. Natl. Acad. Sci. USA 100 (2003) 2450–2455.
[21] C. Haynes, C.J. Oldfield, F. Ji, N. Klitgord, M.E. Cusick, P. Radivojac, V.N. Uversky, M. Vidal, L.M. Iakoucheva, Intrinsic disorder is a common feature of hub proteins from four eukaryotic interactomes, PLoS Comput. Biol. 2 (2006) e100.
[22] J.W. Chen, P. Romero, V.N. Uversky, A.K. Dunker, Conservation of intrinsic disorder in protein domains and families: I. A database of conserved predicted disordered regions, J. Proteome Res. 5 (2006) 879–887.
[23] W. Swietnicki, A.M. Barnie, B.K. Dyas, R.G. Ulrich, Zinc binding and dimerization of Streptococcus pyogenes pyrogenic exotoxin C are not essential for T-cell stimulation, J. Biol. Chem. 278 (2003) 9885–9895.
[24] G. Deleage, B. Roux, An algorithm for protein secondary structure prediction based on class prediction, Protein Eng. 1 (1987) 289–294.
[25] R.D. King, M.J. Sternberg, Identification and application of the concepts important for accurate and reliable protein secondary structure prediction, Protein Sci. 5 (1996) 2298–2310.
[26] J. Garnier, J.F. Gibrat, B. Robson, GOR method for predicting protein secondary structure from amino acid sequence, Methods Enzymol. 266 (1996) 540–553.
[27] Guermer, Y. Ph.D. Thesis.
[28] B. Rost, C. Sander, Prediction of protein secondary structure at better than 70% accuracy, J. Mol. Biol. 232 (1993) 584–599.
[29] D. Frishman, P. Argos, Incorporation of non-local interactions in protein secondary structure prediction from the amino acid sequence, Protein Eng. 9 (1996) 133–142.
[30] J.M. Levin, B. Robson, J. Garnier, An algorithm for secondary structure determination in proteins based on sequence similarity, FEBS Lett. 205 (1986) 303–308.
[31] C. Geourjon, G. Deleage, SOPM: a self-optimized method for protein secondary structure prediction, Protein Eng. 7 (1994) 157–164.
[32] K. Hoffmann, W. Stoffel, TMbase—a database of membrane spanning protein segments, Biol. Chem. Hoppe-Seyler 374 (1993) 166–172.
[33] J.H. Carra, P.L. Privalov, Thermodynamics of denaturation of staphylococcal nuclease mutants: an intermediate state in protein folding, FASEB J. 10 (1996) 67–74.
[34] H. Christensen, R.H. Pain, Molten globule intermediates and protein folding, Eur. Biophys. J. 19 (1991) 221–229.
[35] M. Arai, K. Kuwajima, Role of the molten globule state in protein folding, Adv. Protein Chem. 53 (2000) 209–282.
[36] L. Shi, D.R. Palleros, A.L. Fink, Protein conformational changes induced by 1,1′-bis(4-anilino-5-naphthalenesulfonic acid): preferential binding to the molten globule of DnaK, Biochemistry 33 (1994) 7536–7546.
[37] E. Faudry, G. Vernier, E. Neumann, V. Forge, I. Attree, Synergistic pore formation by type III toxin translocators of *Pseudomonas aeruginosa*, Biochemistry 45 (2006) 8117–8123.
[38] G. Schoehn, A.M. Di Guilmi, D. Lemaire, I. Attree, W. Weissenhorn, A. Dessen, Oligomerization of type III secretion proteins PopB and PopD precedes pore formation in Pseudomonas, EMBO J. 22 (2003) 4957–4967.
[39] L. Zhong, W.C. Johnson Jr., Environment affects amino acid preference for secondary structure, Proc. Natl. Acad. Sci. USA 89 (1992) 4462–4465.